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# Similar regulation of two distinct UL24 promoters by regulatory proteins of equine herpesvirus type 1 (EHV-1)

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ABSTRACT

**To characterise the pattern of the transcriptional regulation of equine herpesvirus type 1 (EHV-1) *UL24* by regulatory proteins, we identified two distinct promoter regions and two transcription initiation (Tci) sites located upstream of the UL24 open reading frame (ORF). The ORF proximal promoter exhibited higher *cis*-activity than that of the distal one. Contrary to the former, the latter performed its function dependent on an initiator (INR) due to its lack of a TATA box. Our results showed that the EHV-1 regulatory proteins EICP0, EICP22 and ETIF *trans*-activated the two promoters, whereas IEP and IR2P displayed negative regulation. In summary, the regulatory proteins exhibited similar regulatory patterns for the two distinct promoters of EHV-1 *UL24*.**



## 1. Introduction

EHV-1 is capable of causing a respiratory disease (rhinopneumonitis) and nervous system damage (myeloencephalopathy) in horses worldwide. Affected equines show a variety of clinical symptoms, including anorexia, fever, ocular discharge, nasal discharge, ataxia of varying severity, paralysis, abortion and neonatal foal death [1,2]. EHV-1 serves as a model for studying aspects of alpha herpesviruses, such as productive and persistent infection, gene regulation and immune responses against these infections with members of this subfamily [3–11]. The genome of EHV-1 harbours at least 76 genes [12,13], including one known as *UL24*. This gene is evolutionarily conserved among many members of the family *Herpesviridae* and is considered as a core gene for herpesviruses [14–16]. The UL24 of herpes simplex virus 1 (HSV-1) exerts some effects on the sub-cellular distribution of viral glycoproteins associated with fusion [17]. It plays an important role in dispersing nucleolin [18]. In addition, it is associated with nervous pathogenicity [19–21]. Transcriptional regulation performs an important role in the pathogenicity of herpesviruses as well as in keeping a cascaded expression of viral genes in the temporal order of immediate-early (α), early (β) and late (γ) upon viral infection [22–24]. The late pattern is further divided into two types: leaky-late (γ1), whose expression begins prior to viral genome replication and subsequently reaches maximal levels after the onset of viral DNA replication and true-late (γ2), whose expression is strictly dependent on replication of the viral genome [25].

The temporal expression of EHV-1 genes is manipulated in a coordinated fashion by seven molecules: an immediate-early (IE) protein, four early proteins (EICP0, EICP22, EICP27 and IR2P), a late ETIF protein and a late IR3 RNA. The IE protein *trans-activates* EHV-1 promoters via binding to 5′-ATCGT-3′ (as a consensus sequence) located in promoters by recruiting more TATA-binding proteins (TBP) and transcriptional factor IIB (TFIIB) to facilitate the formation of the transcription initiation complex [5,7]. IR2P acts as a negative factor to downregulate promoter *cis*-activity by squelching the supplies of limited cellular factors, resulting in dramatically reduced levels of gene expression and viral titres [26,27]. ETIF, a tegument protein, *trans*-activates only the IE promoter by forming complexes with transcription factor Oct-1 that binds to octamer sequences [28,29]. EICP0 independently *trans*-activates all classes of EHV-1 gene promoters by binding to TBP and TFIIB [25,30]. IR3, the RNA antisense to the IE gene, is poorly translated into protein [31]. The biological function of IR3 during EHV-1 infection remains

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unknown [32]. Independently, EICP22 and EICP27 both minimally *trans*-activate promoters. They cooperate synergistically to stimulate the IE gene promoter and together function as co-factors to enhance the activity of IE. EICP27 acts together with EICP0 to *trans*-activate early and leaky-late promoters. Also, EICP27 interacts directly with TBP. However, EICP22 and EICP0 do not function synergistically to affect any EHV-1 promoters [10,33,34].

The thymidine kinase gene (TK) in *Herpesviridae* overlaps the promoter of neighbouring *UL24* on the complementary strand. This genomic range is consistently conserved in many herpesviruses [16,35,36]; for instance, in human herpesvirus 1 (HHV-1), also called HSV-1. The *UL24* of HSV-1 transcripts that contain the entire open reading frame (ORF) are derived from two transcription initiation (Tci) sites. Another initiation site is present within the ORF, which may yield specific mRNA required for translating a truncated form of this protein. These transcripts fall into two groups, dependent on two distinct polyadenylation (polyA) sites; one site is located downstream of the UL24 termination codon and the other is present downstream of the *UL26* ORF [14,37,38]. The *UL24* polyA site-utilising transcripts exhibit early kinetics (β) and those utilising the *UL26* polyA site exhibit leaky-late kinetics (γ1) [14]. The kinetics of HSV-1 UL24 expression can be regulated at a posttranscriptional level by alternative polyadenylation stimulated by HSV-1 ICP27 (an EICP27 homologue) [37,38]. Therefore, *UL24* is considered as a potential model for elucidating ICP27 function. This may apply to the temporal regulation of EHV-1 UL24 expression in consideration of the close genetic relationship between EHV-1 and HSV-1. The sequence identity between EHV-1 and HSV-1 is 48.4% (nucleotide) or 35.2% (amino acid) upon determined by Clustal W alignment. However, both the promoter region and transcriptional regulation by regulatory proteins are unknown for EHV-1 *UL24* as well as for the HSV-1 homologue. Moreover, it is generally accepted that the key determinants of gene expression kinetics are the differences in transcriptional regulation resulting from variations in promoter architecture.

## 2. Materials and methods

### 2.1. Cell culture, EHV-1 growth and titration

Rabbit kidney cells (RK13) were purchased from the American Type Culture Collection (ATCC) and cultured them in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% $CO_2$. The 438/77 strain of EHV-1 were obtained from ATCC and propagated in the RK13 cells and performed the plaque assays to titre EHV-1 as previously described [39].

### 2.2. 5′ Rapid amplification of cDNA ends (RACE)

The total RNA from RK13 cells infected with EHV-1 at 2 multiplicity of infection (m.o.i) was obtained by using an RNA Purification Kit (Fermentas) according to manufacturer's instructions. The 5′RACE was performed using the 5′-Full RACE Kit (TAKARA) and the following UL24-specific primers: UL24 Outer primer and UL24 Inner primer; the primers for adaptor were 5′RACE outer primer and 5′RACE inner primer (Fig. 1A). The 5′RACE polymerase chain reaction (PCR) products were inserted into the pMD18-T cloning vector (TAKARA) and sequenced it using RV-M and M13-47 primers.

### 2.3. PCR and plasmids

All primers used to obtain the *UL24* promoter regions are listed in Table 1, and *Kpn*I and *Bgl* II are both underlined and italicised. The genomic DNA of EHV-1 was prepared according to the Viral DNA Kit (Omega) instructions. We designed two primer pairs to amplify *UL24I* and *UL24II-1* using genomic DNA as a template. To obtain *UL24II-2*, *-3*, *-4*, *-5*, *-6*, *-7* and *-8* (Fig. 2A), we designed a series of different reverse primers and intermediate primers for its respective PCR round using *UL24II-1* as a template. The mutation and deletion of GC box, TATA box and INR in *UL24I* and *UL24II-5* was achieved by sequence overlapping extension (SOE) PCR using aided primers without a TATA box or INR or with mutated GC box, TATA box and INR (Table 1). All the PCR products were purified by using a Gel Extraction Kit (Omega), and digested them with endonucleases *Kpn*I and *Bgl* II followed by a second gel purification for downstream assays.

We previously constructed the effector plasmids expressing regulatory proteins in our lab. We inserted the prepared PCR products for the *UL24* promoter regions into pGL3-Basic (Clon-tech) to produce firefly luciferase reporter plasmids. We confirmed all recombinant plasmids by DNA sequencing before the follow-up experiments.

### 2.4. Transfection

RK-13 cells were plated within 24 h prior to transfection and cultured to a monolayer cell density of 80% confluency in 6- or 24-well plates. The fresh DMEM containing 10% FBS were added into the culture wells. The transfection reagent, PolyJet (Signagen), and plasmids were diluted with serum-free DMEM and high glucose, respectively. The diluted PolyJet was immediately added to the diluted DNA solution all at once followed by immediately mixing and vortexing briefly, and let transfection complex formed at room temperature for 15 min and added onto added drop-wise onto the media and homogenised by swirling gently. To reduce cytotoxicity, the media containing transfection complex were replaced with fresh DMEM containing 10% FBS 6 h post transfection.

### 2.5. Western blot

In our lab, we previously cloned the HA-tagged IEP, IR2P, EICP0, EICP22, EICP27 and ETIF genes into pEF4/myc-His B (Invitrogen) to generate a series of effector plasmids, namely: pIE, pIR2, pEICP0, pEICP22, pEICP27, and pETIF. Western blot was performed exactly as described previously. Proteins from RK13 cells transfected with the above plasmids were isolated in RIPA buffer, separated by SDS–PAGE, and then electro-transferred onto nylon membranes. Subsequently, the membranes were blocked in the TBS containing 5% skimmed milk for 2 h at room temperature, and incubated with the diluted anti-HA tag mouse monoclonal antibody (mAb) and anti-β-actin mouse polyclonal antibody (Sigma) in the 5% skimmed milk for 2 h. Membranes were rinsed three times using TBS containing 0.05% Tween 20 (TBST) and incubated with Alexa Fluor 800-conjuated goat anti-mouse IgG (Odyssey) secondary antibody for 1 h at room temperature. Target proteins were detected by using the Odyssey system (Li-Cor) follow ing a second round of TBST rinsing.

### 2.6. Luciferase reporter assay

We performed the luciferase reporter assays for detecting *UL24* promoter regions with slight modification as previously described [40]. Briefly, RK13 cells cultured in 24-well were transfected with 0.5 μg of a series of reporter plasmids, a negative control (pGL3-Basic) and a positive control (pGL3-Control) together with 0.05 μg of an internal control plasmid pRL-TK expressing a renilla luciferase. The luciferase activities were determined using a dual-luciferase reporter assay system (Promega) and a luminometer

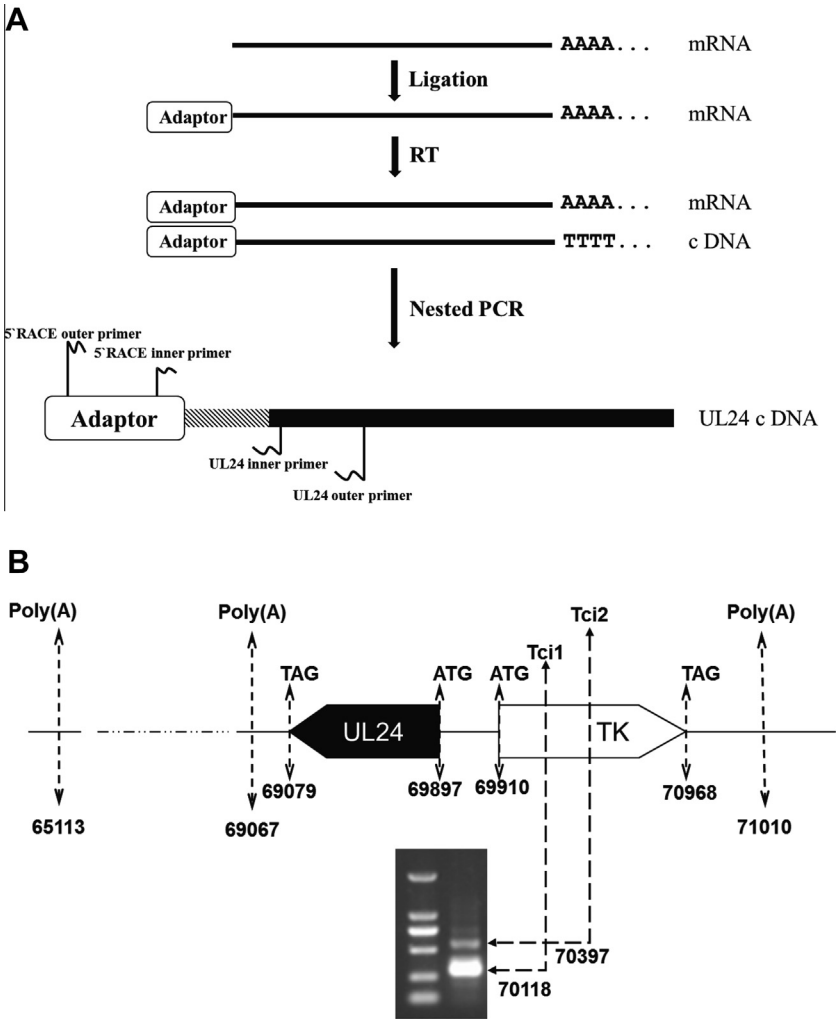


**Fig. 1.** A schematic map of 5′RACE amplification and the EHV-1 *UL24* transcription initiation site. (A). Total RNA was obtained from RK13 cells infected with EHV-1 at 6 hpi. The adaptor was fused to the 5′ terminal end of the mRNA in the presence of T4 RNA ligase after removing the 5′phosphate groups by CIAP, and the 5′cap structure by TAP. At the bottom of this panel, the twill rectangle represents the 5′ untranslated region (5′UTR) between the adaptor and the UL24 ORF. The number above the black rectangle indicates the 5′terminal base locus of the outer and inner primers in the EHV-1 Ab4 genome (Genebank: AY665713). RT, reverse transcription; (B) at the top of this panel, the numbers represent the respective loci of the initiation codon ATG, stop codon TAG and the polyA sites of the *UL24* and *TK* in the EHV-1 genome. The bottom part shows that the inner PCR products were stained with ethidium bromide in a 1.2% agarose gel. The numbers flanking this image indicate the corresponding loci of Tci1 (lower band) and Tci 2 (upper band) in the EHV-1 genome. The other faint bands were not derived from the EHV-1 genome but from RK13 cells upon DNA sequencing and BLAST analysis.

(Berthold). The relative firefly luciferase activity was normalised to renilla luciferase activity in each assay. Each test was repeated in triplicates in one independent experiment, which was performed three times.

To characterise the regulation pattern of EHV-1 regulatory proteins upon *UL24* promoter regions, RK13 cells were co-transfected with 0.3 pmol of promoter reporter plasmids and 0.3 pmol of effector plasmids or empty vectors. The total amount of DNA per transfection was adjusted to 6 μg by the addition of pEF4/myc-His B. To exclude the possibility of regulatory proteins affecting SV40 promoter *cis*-activity, we normalised the firefly luciferase activity; did not use pRL-TK but instead altered the protein concentration of cell lysates as previously described [41].

## 3. Results

### 3.1. Two distinct initiation sites of UL24 transcription

To analyse the transcription initiation sites located upstream of the *UL24* translation start site (ATG), we performed 5′RACE assay using the total RNA of RK13 cells infected with EHV-1 438/77 strain at 8 hpi. The first round of amplification was run by using the outer primer pair. No bands were visible after the first amplification round. The product of the first round of PCR (1 μl) was used as a template to perform the second round of amplification round with the inner primer pair (Fig. 1A). As shown in the lower panel of Fig. 1B, PCR products were identified using 1.2% agarose gel stained with ethidium bromide. No PCR products were obtained from the parallel reactions free reverse transcriptase or when we used water as a template (data not shown), which showed that the PCR products were derived from RNA. Approximately 15–20 clones from each 5′RACE product were sequenced and confirmed to harbour identical 5′ ends, which indicated that they were derived from a single *UL24* mRNA species. The preliminary assay showed the presence of two positive bands of ~500 bp and ~200 bp as well as several interfering DNA bands in the PCR products (Fig. 1B). However, the nature of these interfering bands was unclear, and their sequence data was not significantly similar to the published EHV-1 sequence. Upon sequence alignment, we identified two distinct transcription initiation sites (Fig. 1B, upper panel) located at 70 118 nt (Tci 1) and 70 397 nt (Tci 2) of the EHV-1 genome.

**Table 1**
Primers required for amplifying UL24 promoter regions.

| Name[a] | Primer sequence (5'-3')[b] |
|---|---|
| UL24I.F | AGTTAGATCTTCGGCTGCGAGTGGAGGAAGT |
| UL24I.R | CGAGGTACCGGACATGTCTCCGATGAGGTA |
| UL24I.M.TATA.U | CGGTTCTGGGTGTCGAACCAACCACTAATTACGTCC |
| UL24I.M.TATA.L | GGACGTAATTAGTGGTTGGTTCGACACCCAGAACCG |
| UL24I.Δ.TATA.U | CGGTTCTGGGTGTCGCCACTAATTACGTCC |
| UL24I.Δ.TATA.L | GGACGTAATTAGTGGCGACACCCAGAACCG |
| UL24II-1.F | TCTAGATCTTGTGCGCGCTGATTGCCATGGTTG |
| UL24II-1.R | ACGGGTACCACAATGAGGCGGAGGTTCATATCCACA |
| UL24II-2.R | CCTGGTACCCGTGATGCCCGTTTG |
| UL24II-3.R | ATAGGTACCCGAAGAAGATGGGAG |
| UL24II-4.R | ATTGGTACCGGTGCTGCTCATGAGGG |
| UL24II-5.R | GCGGGTACCTCCAATATCACTCCA |
| UL24II-5.ΔINR.F | TCTAGATCTTGTGCTGCCATGGTTGCCACCCTA |
| UL24II-5.M.INR.F | TCTAGATCTTGTGCCCCGGCCCTGCCATGGTTG |
| UL24 Outer primer | TCGCTTGGCGCTTGCCAGGTCTTGA |
| UL24 Inner primer | ACCAGCTCTGAGTCTGCTTCTAGCT |
| 5'RACE Outer primer | CATGGCTACATGCTGACAGCCTA |
| 5'RACE Inner primer | CGCGGATCCACAGCCTACTGATGATCAGTCGATG |
| UL24I.M.GC.U | ATAAGGTAGGTTGTGGTAAAGC |
| UL24I.M.GC.L | GCTTTACCACAACCTACCTTAT |
| UL24II-5.M.GC.U | CGATTCTGGCTTGGGTGCGCAG |
| UL24II-5.M.GC.L | CTGCGCACCCAAGCCAGAATCG |
| UL24II-6.F | AGTAGATCTCTGGGCGCTTGAC |
| UL24II-7.F | AACGGGAGATCTGGTCATGGGGTGC |
| UL24II-8.U | GCACCCCATGACGCGTGAACTTC |
| UL24II-8.L | GAAGTTCACGCGTCATGGGGTGC |

[a] The primers appended with letter "F" or "R" were used to obtain the end-product of target DNA sequence, F: forward, R: reverse; the oligos with letter "U" or "L" were adopted to amplify the intermediate product for SOE PCR, U: upper, L: lower. UL24 Outer and Inner primer was designed based on EHV-1 Ab4 strain genome. 5′RACE Outer and Inner primer was provided in the 5′RACE kit.
[b] The underlined 6 nucleotides represented Kpn *I* (GGTACC) and Bgl *II* (AGATCT) sites. The boxed nucleotides were the mutated sites in the TATA box, INR and GC box of *UL24I* and *UL24II-5*.

### 3.2. Identification of UL24 gene promoter regions

To identify the promoter regions of EHV-1 *UL24*, we designed a series of primers to obtain six DNA fragments upstream of *UL24* using genomic DNA as a template (Table 1). Each promoter region was mapped based on an individual Tci (+1) position (Fig. 2A). In these chosen promoter regions, the 3′ flanking sequence downstream of the Tci did not contain ATG to avoid interfering with luciferase gene translation. In order to evaluate the putative promoter *cis*-activity of these regions, a set of luciferase reporter plasmids were engineered by cloning the above fragments into pGL3-Basic (promoter- and enhancer-less) as well as itself as negative control. Each reporter plasmid was transiently transfected into RK13 cells together with the internal control plasmid pLR-TK. Firefly luciferase activity was used to assess putative promoter activity according to the dual luciferase reporter protocol instructions. As shown in Fig. 2B, maximal activity was observed 48 h post-transfection in RK13 cells transfected with reporter plasmid containing *UL24I*. As compared with the negative control, *UL24II-1* and *UL24II-5* showed higher activity, with the highest activity in the former. In contrast, *UL24II-2*, *UL24II-3* and *UL24II-4* had lower activity than *UL24II-1* or *UL24II-5* and displayed the same activity level as negative control yet. Meantime, *UL24II-7* displayed higher activity than *UL24II-1*, *UL24II-5*, *UL24II-6* and *UL24II-8*. *UL24II-5* and *UL24II-7* had a promoter activity and can be downregulated by the fragment located between them; *UL24II-8*, a fusion fragment of *UL24II-5* and *UL24II-7*, did not exhibited higher level than each one but just as did the negative control (Fig. 2C). These findings indicated that *UL24I* and *UL24II-5* are responsible for *UL24* transcription initiation at Tci 1 and Tci 2, respectively.

### 3.3. Functional initiator, TATA box and GC box in the UL24 promoter

We identified two fragments upstream of the UL24 translation start site as promoters. Upon common location of TATA box, initiator (INR) [42] and GC box [43] in the promoter region combined with computational analysis [44], a putative TATA box and GC box were located 24 nt and 107 nt upstream of Tci 1 and a putative INR was obtained from Tci 2 as well as GC box at +110 (Fig. 3A upper panel). The HSV-1 *UL24* Tcis were identified in HSV-1 strain 17 genome: 47 402 nt and 47 666 nt [14]. To evaluate how conserved the initiation sites and relevant motifs are between EHV-1 and HSV-1, we selected EHV-1 70 536–70 048 nt covering two Tcis and *cis*-elements) and HSV-1 47 272–47 736 nt followed by sequence comparison. It is little known on HSV-1 *UL24* promoter region as well as relevant *cis*-elements. Thus, its GC box, TATA

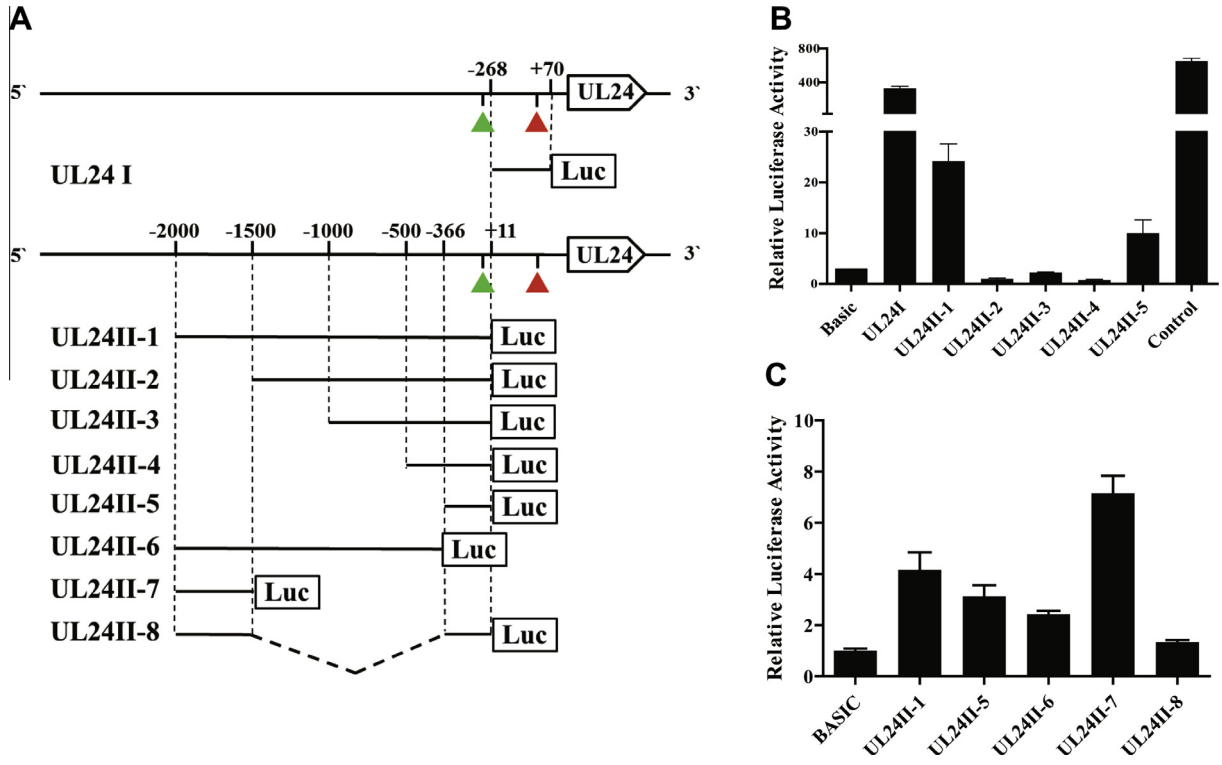


**Fig. 2.** Identification of the *UL24* promoter region using a luciferase activity reporter assay. (A) Each transcription site was set as +1, Tci 1 (red triangle) and Tci 2 (green triangle). Each DNA fragment was obtained using PCR with the primers listed in Table 1. (B) The *UL24I*, *UL24II-1* and *UL24II-5* exhibited promoter activity comparing with the negative control (pGL3-Basic) in the following order: *UL24I* > *UL24II-1* > *UL24II-5*, but *UL24II-2*, *3* and *4* did not. As expected, the positive control (pGL3-Control) displayed the highest luciferase activity. (C) The promoter activity appeared in the order: *UL24II-7* > *UL24II-1* > *UL24II-5* > *UL24II-6* > *UL24II-8* (similar to pGL3-Basic). The *UL24II-8* was constituted of *UL24II-5* and *UL24II-7*. The firefly luciferase activity was normalised to renilla luciferase activity as an internal control. One representative of three performed experiments is presented [mean ± standard deviation (S.D.)].

box and INR were predicted upon the above mentioned methods. It was observed that their loci and nucleotides in promoter region were almost one-to-one correspondence between EHV-1 *UL24* promoter and HSV-1 *UL24* promoter regions (Fig. 3A lower panel). To determine whether the GC box, TATA box and INR were contributing to the *UL24I* and *UL24II-5* promoter activity, we deleted or mutated these *cis*-acting elements using *UL24I* and *UL24II-5* with primers (Table 1). As shown in Fig. 3B and C, the mutation or deletion of the TATA box (*UL24I*Δ TATA and *UL24I*.M.TATA), INR (*UL24II-5*Δ INR and *UL24II-5*.M.INR), and GC box (*UL24I*.M.GC and *UL24II-5*.M.GC) reduced firefly luciferase activity, respectively, indicating that *UL24* promoters have the functional TATA box, INR and GC box and are indispensable to promoter activity in RK13 cells.

### *3.4. Distinct regulation of UL24 promoters*

The ectopic expression of six regulatory proteins was shown by Western blot analysis at 48 h post-transfection into RK13 cells (Fig. 4A). To characterise regulation of the *UL24* promoters by the EHV-1 regulatory proteins IEP, IR2P, EICP0, EICP22, EICP27, and ETIF, luciferase assays were performed with a set of regulatory protein expression plasmids. As shown in Fig. 4B, EICP22 increased *UL24I* and *UL24II-5* promoter activity by 2.4-fold and 6-fold, respectively, as compared with luciferase activity achieved in RK13 cells co-transfected with empty vector. Moreover, under the same conditions, EICP0 increased *UL24I* and *UL24II-5* promoter activity by 2.3-fold and 17.5-fold, respectively; ETIF also did it by 1.8-fold and 4-fold, respectively. In contrast, IR2P reduced *UL24I* and *UL24II-5* promoter activity by 43.4-fold and 26.3-fold, respectively; IEP also reduced promoter activity by 2.3-fold and 3.9-fold, respectively. EICP27 regulated *UL24I* and *UL24II-5* promoter activity by <1.49 fold, which was considered an extremely small effect. These results showed that EHV-1 regulatory proteins had different effects on *UL24I* and *UL24II-5* promoter activity and that *UL24II-5* is more sensitive than *UL24I* to regulation exerted by regulatory proteins except for EICP27. Moreover, *UL24II-1*, *UL24II-2*, *UL24II-3*, *UL24II-4* and *UL24II-5* exhibited nearly same regulatory pattern exerted by regulatory proteins.

## 4. Discussion

In this study, we identified the exact two promoter regions required for EHV-1 *UL24* transcription at two distinct Tci sites located upstream of the UL24 ORF. Our results showed that the regulatory patterns of six EHV-1 regulatory proteins for the two promoters were very similar. EICP22, EICP0 and ETIF played a positive role, whereas IEP and IR2P played a negative role. EICP27 did not play any part in this context. This information might provide references for further investigation of transcriptional regulation of UL24 homologues of the *Herpesviridae* family.

As shown in Fig. 2B and C, *UL24II-7* displayed higher activity than *UL24II-1* and *UL24II-6*, and *UL24II-5* did so than *UL24II-2*, *3*, *4*. This result suggested that the sequence range from −1500 and −366 nt upstream of Tci 2 functioned as a negative element and exerted negative regulatory effect on *UL24II-5* and *UL24II-7* promoter activity. *UL24II-2*, *3*, *4* containing this negative sequence just only displayed the similar activity to negative control. However, *UL24II-6* possessed superior capability to negative control even overlapping this negative element. The *UL24II-8* constituted of *UL24II-5* and *UL24II-7* just displayed the negative control level, which demonstrated that *UL24II-7* did not directly assist *UL24II-5*

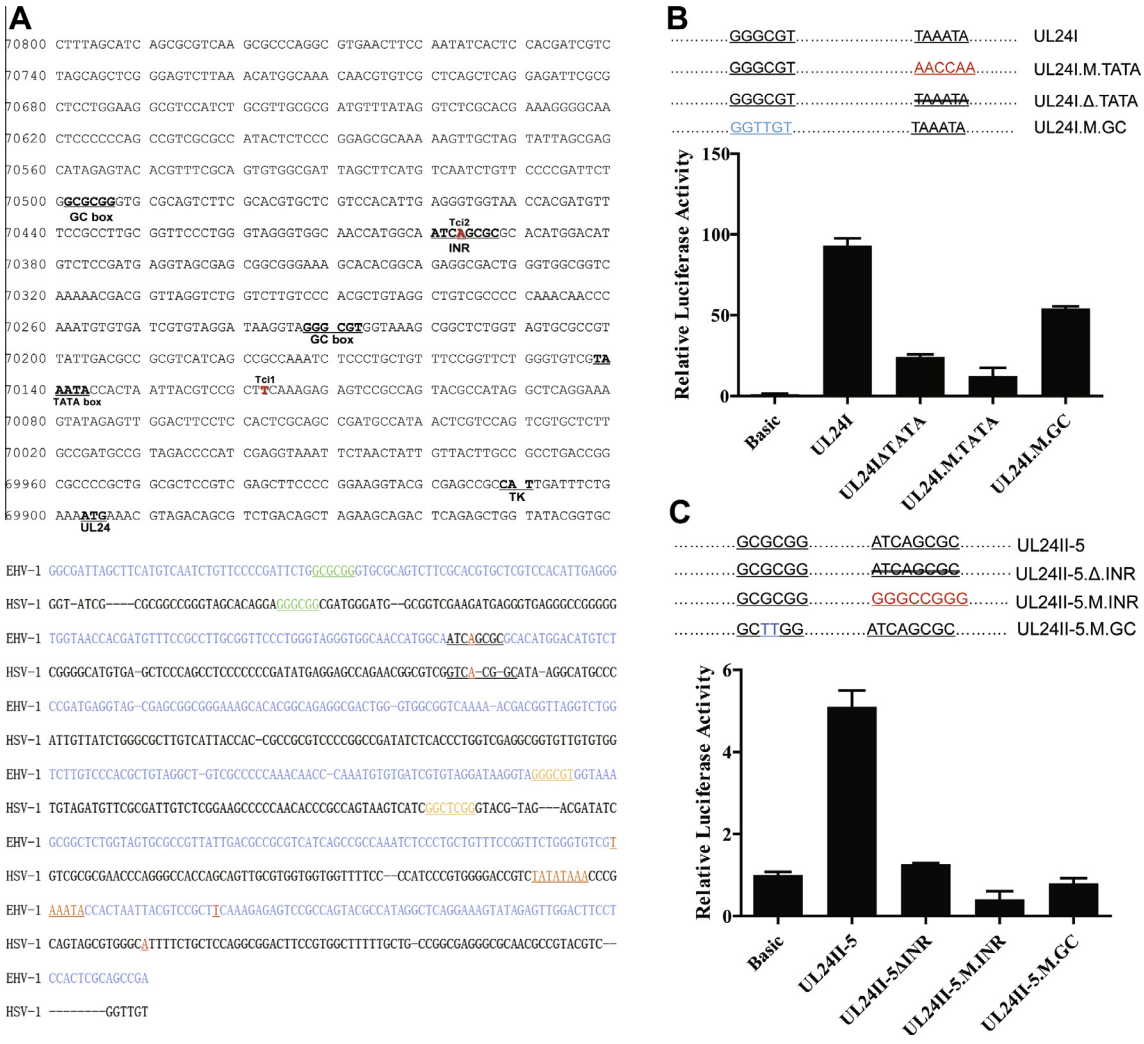


**Fig. 3.** The functional TATA box, INR and GC box in the *UL24* promoter region. (A) The TATA box, INR GC box and Oct-1 site were predicted and analysed by GPMiner [44] or JASPAR [57] combined with published references [28,29,42,43]. Upper panel: These *cis*-elements, start codon ATG of *UL24* and its complement format CAT of *TK* were underlined in the upstream sequence of the *UL24*. The transcription initiation sites were detected by 5′RACE and marked with red letter and indicated as Tci (transcription initiation site); lower panel: HSV-1 UL24 Tcis was represent ed as red letter and reported in the reference [14]; the sequence comparison between EHV-1(70536–70048 nt) and HSV-1 (47272–47736 nt) were performed with MegAlign. (B) *UL24I* mutants represented by *UL24I*.Δ.TATA (deleted TATA box), *UL24I*.M.TATA (mutated TATA box) and *UL24I*.M.GC (mutated GC box) were shown in the upper panel. These mutants displayed lower activity than *UL24I* in the lower panel. (C) *UL24II-5* mutants represented by *UL24II*-5.Δ.TATA (deleted TATA box), *UL24II*-5.M.TATA (mutated TATA box) and *UL24II-5M.GC* (mutated GC box) were shown in the upper panel. These mutants displayed lower activity than *UL24II-5* in the lower panel. The firefly luciferase activity was normalised to renilla luciferase activity as an internal control. One representative of three performed experiments is presented (mean ± S.D.).

in initiating transcription. These results indicated that *UL24II-7* was capable of counteracting the inhibitory effects of the negative sequence and indirectly aiding *UL24II-5* to ensure *UL24* transcription from Tci 2. Therefore, it could be hypothesised that *UL24II-7* would generate a novel transcript possibly in term of upon promoter activity. In the future research, more work needs to be conducted on detecting the novel transcript and its function, and the exact mechanism underlying that *UL24II-7* performs remote regulation on *UL24II-5* promoter activity.

Robertson and Whalley analysed the same genome context sequence (*UL24*/*TK*) of EHV-1, comparing the EHV-1 *TK* with that of HSV-1 and other herpesviruses and assigned putative core *cis*-elements in TK promoter. Furthermore, it was proposed that the functional verification should be required to identify the potential TATA box determining the Tci of TK transcript [45]. Therefore, to better explore molecular data as well as figure out possible UL24 promoter structures, we adopted the bioinformatics and referred to the relevant literature and data to predict the TATA box, GC box and INR, followed by functional identification based on site-directed mutation and deletion. GC box has a consensus sequence GGGCGG and performs function based on its position in the promoter region but is not orientation independent. Usually, it is located in the sequence upstream of the TATA box and approximately 110 bases upstream from the transcription initiation site. The GC elements are bound by transcription factors and have similar functions to enhancers [43]. The mutated or absent GC box can evidently downregulate *UL24I* and *UL24II-5* as well as the mutated or absent TATA box and INR do so (Fig. 3B and C). Thus, the GC box, TATA box and INR are functional for initiating *UL24* transcription. Pearson and Coen reported that the transcription start sites of HSV-1 *UL24* is 47402 nt and 47666 nt of HSV-1 strain 17 genome [14]. In this study, the GC box, TATA box and INR in HSV-1 UL24 promoter were predicted and compared with EHV-1 counterparts (Fig. 3A lower panel). It has been observed that their loci and nucleotides in promoter region are almost one-to-one correspondence. However, it remains unknown whether or not the HSV-1 UL24 promoter has a similar regulatory pattern to EHV-1 counterpart.

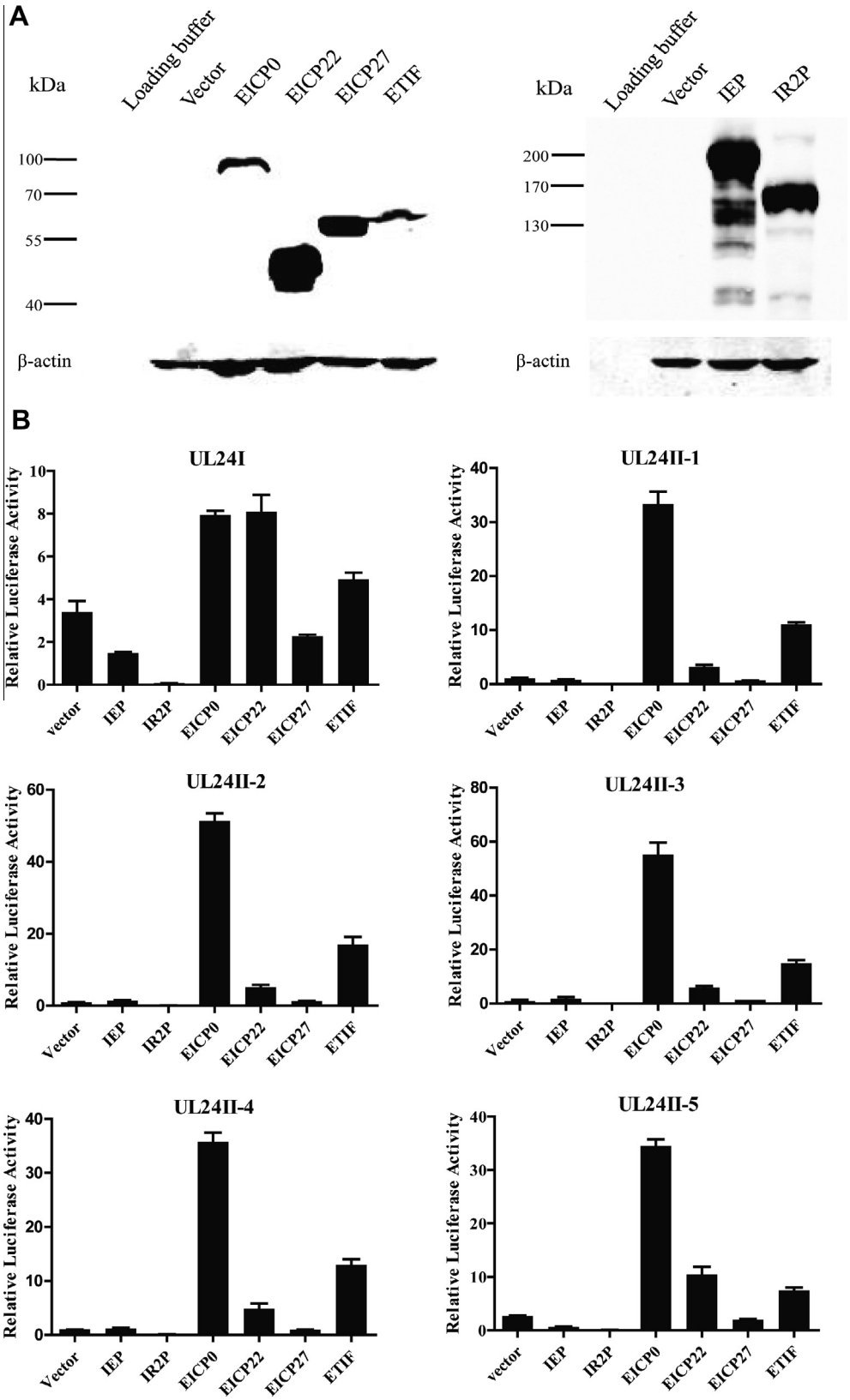


**Fig. 4.** The regulatory effects of the EHV-1 regulatory proteins on the *UL24* promoter regions. (A) All six proteins were expressed in RK13 cells and detected by Western blotting. (B) EICP0, EICP22 and ETIF *trans*-activated *UL24I* and *UL24II-5*; IEP and IR2P inhibited these two promoters' activity; EICP27 had hardly any effects on *UL24I* and *UL24II-5*. *UL24II-1*, *2*, *3* and *4* presented the same pattern as *UL24II-5* under regulation exerted by EHV-1 regulatory proteins. Firefly luciferase activity was normalised to the protein concentration of the cell lysates as described previously [41]. The luciferase activities are presented as means ± S.D. of three replicates per treatment in one assay of three independent experiments.

The appropriate initiation of gene transcription is a necessary prerequisite for adequate translation and subsequent functions in the life cycle of a virus. This may be particularly important for EHV-1 and all the *Herpesvirales* during infection and/or replication in vivo and in vitro. This is due to the huge size of the herpesvirus genome as well as the fact that it harbours overlapping genes such as *IE/IR2* [26,27], *IE/IR3* [31], *UL24/TK* [35], *UL26.5/UL26* [12], UL49.5/UL50 [46], *ICP18.5/gB* [47] and *LAT/ICP0* [48,49]. The transcription and expression of herpesvirus genes occurs in an ordered fashion of three cascaded stages: immediate-early, early and late. Regulatory proteins control these stages, which may be a strategy to ensure that herpesviruses utilise the hosts' resources most efficiently and overcome physical constraint among contiguous genes and overlapping genes in their genomes of a limited size.

Dual- or triple-initiation of transcription may be another complicated approach taken by herperviruses, represented by *UL24* [14,50], *ICP22* [51] and *ICP27* [52]. There was detailed information about the transcription start sites, promoter regions and transcriptional regulation of the latter two. However, it is not known why herpesviruses endow them with two transcription start sites located upstream of their respective ORF. In this study, we focused on the transcriptional regulation of *UL24* mainly due to the following perspectives: (i) it is highly conserved throughout the whole *Herpesvirales*; (ii) its transcription frequency is closely related with *TK* transcription; (iii) UL24 is an important virulence determinant associated with neuropathogenicity; (iv) there was previously no information about the *UL24* transcription start sites, promoters or transcriptional regulation of most herpesvirus.

Six EHV-1 regulatory proteins were successfully expressed in RK13 cell (Fig. 4A). Although the immunoreactive bands exhibited the higher molecular weights than theoretical value (EHV-1 Ab4 strain: IEP, 155kDa; IR2P, 124kDa; EICP0, 59kDa; EICP22, 32kDa; EICP27, 51kDa; ETIF, 54kDa), the similar results were also reported in previous studies [53–55]. This could be likely due to post-translational modification such as phosphorylation and/or glycosylation mentioned in these references. Another possibility is that these regulatory proteins could be associated with transcriptional factors via protein–protein interaction.

Previous studies reported that the regulatory protein ICP27 played an important role in post-transcriptional regulation and participated in switching from the proximal to distal polyadenylation site at the 3′-end of the *UL26* ORF [36,37]. This resulted in UL24 expression throughout early and late times post-infection [38]. Our results showed that ICP27 does not affect *UL24* promoter *cis*-activity, i.e., it performs no regulatory function on the transcription initiation of *UL24*. As expected, IE does not *trans*-activate the two promoters because there are no IEP-binding sites consensus sequences (5′-ATCGT-3′) properly located near the transcription initiation sites. In this context, IEP acts more like a negative factor similar to IR2P; however, its impact is significantly lower on the latter. IR2P serves as a negative regulator that squelches the limited amount of transcriptional factors required for gene transcription. Intracellular overexpression of IR2P dramatically reduces viral titres in infected cells [26,27]. In this study, EICP0 independently *trans*-activated UL24I promoter activity, consistent with previous findings that EICP0 activates all classes of EHV-1 promoters harbouring a small promoter region containing only a TATA box and cap site [11,30]. This is because EICP0 specifically interacts directly with the basal transcription factors TBP and TFIIB. Even if UL24II-5 lacks a TATA box, it harbours INR that can recruit TBP-associated factors (TAFs) [56] and directly interacts with TBP subsequently forming protein–protein interactions with TFIIB. This may explain why EICP0 independently upregulates UL24II-5 promoter activity in the absence of TATA box.

Our results showed that EICP22 can increase the *cis*-activity of the two promoters of the *UL24* by 2.4-fold and 6-fold. However, EICP22 did not perform the normal functions of an accessory regulatory protein for EICP27 and IE (unpublished data). It may be because neither *UL24I* nor *UL24II-5* is an immediate early promoter and IE cannot *trans*-activate these two promoters. Moreover, we have confirmed that EICP22 cannot interact with TBP directly (unpublished data). The functions of EICP22 remain to be studied further. Thus, *UL24* should serve as a helpful model to elucidate EICP22 functions with novel insights. ETIF can upregulate *UL24I* and *UL24II-5* by 1.8-fold and 4-fold, respectively. However, we did not find regulatory octamer sites (TAATGARAT) within the two promoters or other octamer sequences by computational analysis [57]. One possibility is that ETIF requires another mechanism to *trans*-activate the two promoters rather than forming a complex with Oct-1.

We conclude that EHV-1 *UL24* transcription begins at two start sites upstream of the UL24 ORF subject to being driven by two distinct promoters. The UL24 ORF-proximal promoter has much more *cis*-activity than the distal one. Six regulatory proteins exert similar regulation effects on these two promoters. This suggests that the *UL24* promoter architecture would play a leading role in causing the difference of transcription initiation frequencies from two distinct Tci under the same regulatory conditions. Meanwhile, it is unknown whether or not the two sites for transcription initiation switch mechanically during the EHV-1 life cycle. However, the complicated kinetics of *UL24* mRNA accumulation and its transcriptional profile during EHV-1 infection makes it difficult to directly answer this question.

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